Demethylation of the PD-1 Promoter Is Imprinted during the Effector Phase of CD8 T Cell Exhaustion

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ABSTRACT

PD-1 is an inhibitory receptor that has a major role in T cell dysfunction during chronic infections and cancer. While demethylation of the PD-1 promoter DNA is observed in exhausted T cells isolated from chronically infected individuals, little is known about when this stable demethylation of PD-1 promoter DNA is programmed during the course of a chronic infection. To assess if PD-1 promoter DNA demethylation is impacted by prolonged stimulation during effector phase of chronic infection, we adoptively transferred virus-specific day 8 effector CD8 T cells from mice infected with lymphocytic choriomeningitis virus (LCMV) clone 13 into recipient mice that had cleared an acute infection. We observed that LCMV-specific CD8 T cells from chronically infected mice maintained their surface expression of PD-1 even after transfer into acute immune mice until day 45 posttransfer. Interestingly, the PD-1 transcriptional regulatory region continued to remain unmethylated in these donor CD8 T cells generated from a chronic infection. The observed maintenance of PD-1 surface expression and the demethylated PD-1 promoter were not a result of residual antigen in the recipient mice, because similar results were seen when chronic infection-induced effector cells were transferred into mice infected with a variant strain of LCMV (LCMV V35A) bearing a mutation in the cognate major histocompatibility complex class I (MHC-I) epitope that is recognized by the donor CD8 T cells. Importantly, the maintenance of PD-1 promoter demethylation in memory CD8 T cells was coupled with impaired clonal expansion and higher PD-1 re-expression upon secondary challenge. These data show that the imprinting of the epigenetic program of the inhibitory receptor PD-1 occurs during the effector phase of chronic viral infection.

IMPORTANCE

Since PD-1 is a major inhibitory receptor regulating T cell dysfunction during chronic viral infection and cancers, a better understanding of the mechanisms that regulate PD-1 expression is important. In this work, we demonstrate that the PD-1 epigenetic program in antigen-specific CD8 T cells is fixed during the priming phase of chronic infection.

CD8 T cells play a critical role in controlling acute viral infections and, upon control, can establish antigen-specific memory that provides the host with long-lived immunity to the previously experienced pathogen. While chronic pathogens also generate robust CD8 T cell responses, prolonged exposure to high levels of antigen results in the decline of CD8 T cell effector functions. The progressive decline of effector function in antigen-specific CD8 T cells is coupled with reduced proliferative potential and repression in the ability to express the cytokines interleukin 2 (IL-2), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) upon antigenic restimulation (1, 2). This progressive decline in T cell effector function, known as T cell exhaustion, poses a major challenge for controlling chronic diseases, including HIV, hepatitis C virus (HCV), and HBV infections (3) and cancer (4–6). Thus, there is considerable interest in developing therapeutic strategies to reverse T cell exhaustion for enhancing antipathogen and antitumor CD8 T cell immunity.

Recent investigation into the molecular mechanisms regulating CD8 T cell exhaustion has revealed that expression of inhibitory receptors is causal in the maintenance of the exhausted state (7). Specifically, programmed cell death 1 (PD-1) is the major characterized inhibitory receptor and has been shown to inhibit T cell proliferation as well as reduce the effector function of antigen-specific CD8 T cells. The impact of PD-1 signaling on T cell exhaustion and the resulting implications for cancer immunother-

apy came from studies in which PD-1 signals were blocked in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) (8). Treatment of chronically infected mice with a PD-1 blocking antibody resulted in the expansion of antigen-specific CD8 T cells that had increased effector functions. These findings were immediately extended to several chronic human viral infections, such as HIV, HCV, and many different cancers, including melanoma and lung, bladder, kidney, and head and neck cancers (9–18). The rapid translation of this fundamental discovery into a therapeutic strategy for treating chronic infections and cancer further highlights the untapped potential for PD-1-targeted immunotherapy.

Given the central role that PD-1 plays in current cancer immunotherapy strategies, a better understanding of the transcriptional regulatory mechanisms that govern PD-1 expression is required.

Many labs have shown that upregulated expression of PD-1 on virus-specific CD8 T cells is maintained during chronic infection, while PD-1 is rapidly downregulated on antigen-specific CD8 T cells after viral clearance during an acute infection (8, 19). Accordingly, it is generally accepted that initial upregulation, as well as continued expression of PD-1, is predominantly driven by T cell receptor (TCR) signaling. The molecular mechanisms regulating PD-1 expression were recently explored by examining the roles of transcription factors, including NFAT, FoxO1, T-bet, and Blimp-1, in controlling PD-1 transcription (20–24). Both NFAT and FoxO1 serve as transcriptional activators of the PD-1 promoter by direct binding (22, 24). Blimp-1 also directly binds to the PD-1 locus but represses PD-1 expression in the early phase of acute infection, while T-bet represses PD-1 during chronic infection (20, 21, 23). Along with transcription factors, epigenetic mechanisms play a major role in controlling gene expression, mediating stable maintenance of transcriptional programs. We have recently reported that the PD-1 transcriptional regulatory region underwent dynamic change in epigenetic programming during the development of functional memory and exhausted CD8 T cells. Specifically, we observed that functional memory CD8 T cells reacquired a methylated PD-1 promoter while the PD-1 promoter remained unmethylated in exhausted CD8 T cells even when viral loads were reduced to undetectable levels (25, 26). These reports suggest that long-term antigen exposure enforces the demethylation of the PD-1 promoter in fully exhausted CD8 T cells. However, it remains unclear when stabilization of PD-1 promoter methylation programming sets in during chronic infection. To address the stabilization of effector-phase DNA methylation changes, we used the mouse model of LCMV infection to track the methylation state of the PD-1 promoter in antigen-specific effector CD8 T cells adoptively transferred from chronically infected mice into acutely infected animals. Here we report that effector CD8 T cells generated from a chronic viral infection maintain the demethylated state of the PD-1 locus even when they are transferred and rested in immune recipient mice. Additionally, maintenance of the demethylated locus on virus-specific CD8 T cells is associated with heightened PD-1 re-expression and decreased clonal expansion during a secondary response.

MATERIALS AND METHODS

Mice and infection. Wild-type (WT) C57BL/6 mice were purchased from The Jackson Laboratory. Lymphocytic choriomeningitis virus (LCMV) gp33-41 specific TCR transgenic mice (P14) were used. Mice were infected with LCMV Arm (2×10^5 PFU intraperitoneally [i.p.]), clone 13 (CL13) (2×10^4 or 2×10^6 PFU intravenously [i.v.]), or CL13 V35A (2×10^4 i.v.). All mice were used in accordance with National Institutes of Health and the Emory University Institutional Animal Care and Use Committee guidelines.

Adoptive transfer. A total of 5×10^3 P14 (Thy1.1/1.1 or Ly5.1/5.1) cells were adoptively transferred into congenically distinct B6 mice (Thy1.2/1.2 or Ly5.2/5.2). P14 chimeric mice were then infected with LCMV Arm (2×10^5 PFU i.p.) or CL13 (2×10^6 PFU i.v.). CD8 T cells were purified from spleen using a CD8 T cell purification kit (Miltenyie Biotech) at day 8 postinfection. A total of 2×10^6 (Thy1.1/1.1 or Ly5.1/5.1) P14 cells from LCMV Arm-infected P14 chimeric mice and 2×10^6 (Thy1.1/1.2 or Ly5.1/5.2) P14 cells from LCMV CL13-infected P14 chimeric mice were cotransferred into infection-matched (day 8 LCMV Arm infection) host mice. To examine the recall response, sorted 5×10^3 memory P14 cells derived from acute P14 (Ly5.1/5.1) cells and chronic P14 cells (Ly5.1/5.2) were cotransferred into naive B6 mice, followed by LCMV Arm (2×10^5 PFU i.p.) infection.

Flow cytometry and staining. Lymphocytes were collected from peripheral blood mononuclear cells (PBMCs), spleen, and liver, as previously described (27). LIVE/DEAD Near-IR (Invitrogen) was used to remove the dead cells. For intracellular cytokine staining, splenocytes were incubated with gp33-41 peptide (0.2 μg/ml) for 6 h at 37°C in the presence of brefeldin and monensin. Cells were stained with anti-IFN-γ, anti-TNF-α, and anti-IL-2 antibodies (Abs) (BD) using Cytofix/Cytoperm (BD) according to the manufacturer's recommendations. Cells were analyzed on a Canto II flow cytometer (BD Immunocytometry Systems). Data were analyzed with FlowJo v.9.2 (TreeStar).

Genomic DNA methylation analysis. Genomic DNA was purified from sorted P14 cells at day 30 posttransfer. Bisulfite-induced deamination of cytosine was performed using the Zymo Research EZ DNA methylation kit. This bisulfite-modified DNA was amplified by PCR with locus-specific primers as previously described (25).

RESULTS

LCMV-specific effector CD8 T cells from chronic infection maintain surface PD-1 expression after transfer into acute immune mice. We have previously reported that demethylation of both the mouse and human PD-1 promoters is coupled to PD-1 expression during effector differentiation of virus-specific CD8 T cells (25). Furthermore, the demethylated state of the PD-1 promoter persists in CD8 T cells that have prolonged exposure to antigen during chronic infections. Surprisingly, we found that the demethylated state continued even when virus levels were reduced and PD-1 expression was downregulated (25). In this study, we sought to further understand the direct relationship between duration of antigen exposure and maintenance of the demethylated PD-1 promoter in virus-specific CD8 T cells. To investigate the relationship between stability in DNA methylation programming and antigen persistence, we utilized the lymphocytic choriomeningitis virus (LCMV) mouse model of acute and chronic viral infections. This model allows for the examination of effector and memory differentiation of antigen-specific CD8 T cells that develop in response to either acute or chronic antigen presentation. Furthermore, adoptive-transfer experiments using P14 TCR transgenic CD8 T cells that recognize a dominant LCMV epitope (gp33-41) facilitates tracking of phenotype, function, and epigenetic programs longitudinally in mice that had cleared an acute LCMV infection. To track the plasticity of the DNA methylation program during LCMV infection, we generated chimeric mice by adoptively transferring congenically distinct naive P14 cells into WT animals. The chimeric animals were then infected with the acute or chronic strain of LCMV. At 8 days postinfection, equal numbers of "acute" and "chronic" effector P14 cells were cotransferred into infection-matched mice that were infected with the acute strain of LCMV. Thus, the existing effector pool of CD8 T cells in the recipient mice were capable of clearing any residual virus that was potentially transferred with the effector P14 cells from LCMV CL13-infected mice (Fig. 1a).

To first assess the ability of donor chronic P14 cells to persist after transfer, we longitudinally measured the number of acute and chronic P14 cells in the blood of recipient mice until 45 days after adoptive transfer. Chronic P14 cells underwent a much greater level of contraction than did acute P14 cells between days 1 and 8 posttransfer, but following the contraction phase of the immune response, the ratio of chronic P14 cells among total P14 cells was maintained at a steady frequency (~20% of total P14) until day 45 posttransfer (Fig. 1b). We next examined changes in PD-1 expression on acute and chronic P14 cells in the blood of
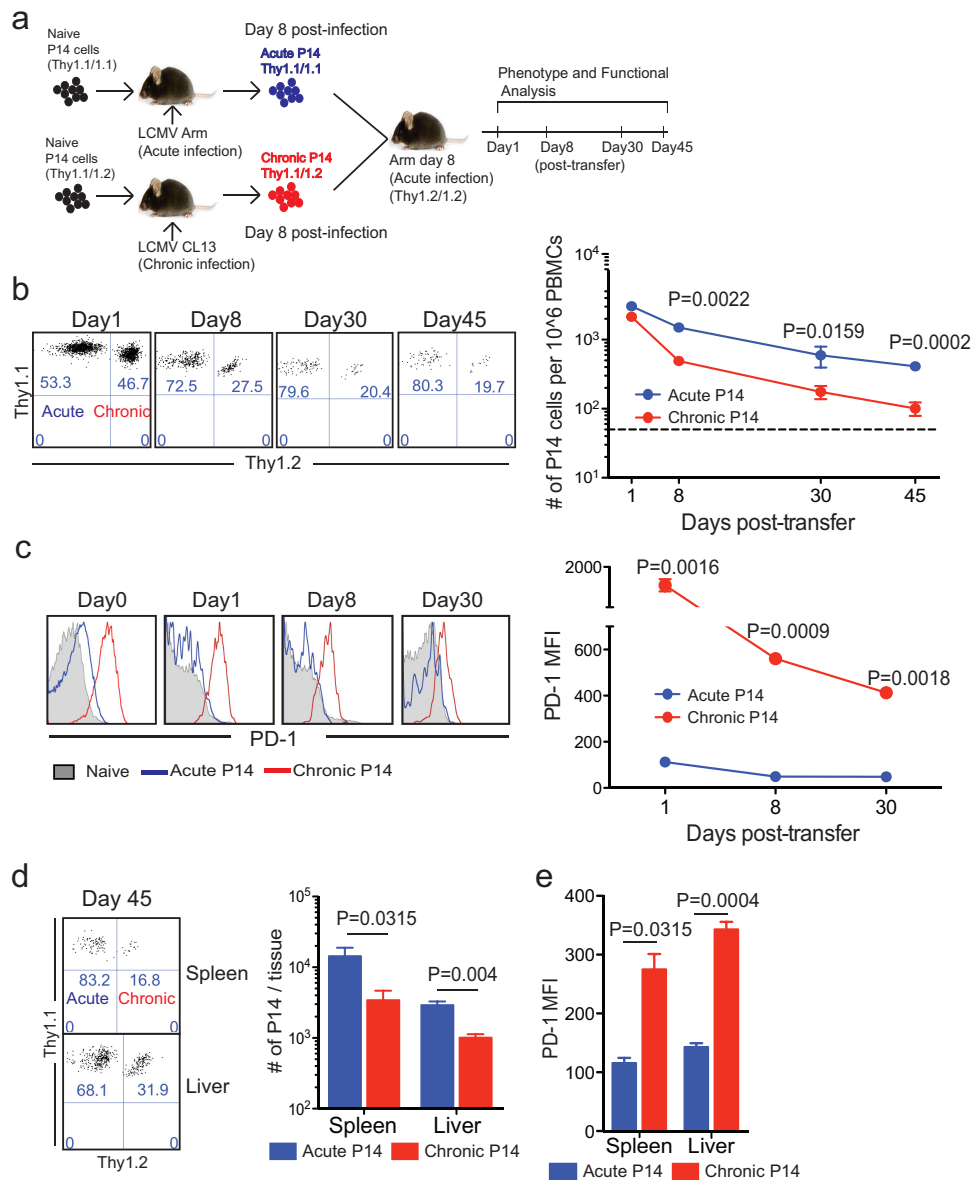
FIG 1 LCMV-specific day 8 P14 cells from chronically infected mice show sustained expression of PD-1 after transfer into acute immune mice. (a) P14 cells from either LCMV Arm or CL13 infection were cotransferred into infection-matched (Arm day 8) mice at day 8 postinfection. The phenotype and function of P14 cells were analyzed at days 1, 8, 30, and 45 posttransfer. (b) The ratios of acute and chronic P14 cells among total P14 cells in blood are shown on the left. The numbers of acute and chronic P14 cells in blood were examined at days 1, 8, 30, and 45 days posttransfer and are shown on the right. (c) Expression of PD-1 on acute and chronic P14 cells at days 1, 8, and 30 posttransfer is shown on the left. A summary graph is shown on the right. (d) The ratios of acute and chronic P14 cells in spleen and liver were examined at day 45 posttransfer. A summary graph is shown on the right. (e) Expression levels of PD-1 were analyzed in spleen and liver at day 45 posttransfer. Data are representative of results from two or three independent experiments with three to five mice per group. Error bars show SEM.

acute immune mice. Since the acute strain of LCMV is controlled to undetectable levels by day 8 postinfection, donor P14 cells from the acutely infected mice had already low levels of the PD-1 expression. In contrast, donor P14 cells isolated from the chronically infected mice had high levels of PD-1 expression (Fig. 1c, day 0). Upon transfer into the infection-matched recipient mice, the PD-1 expression on the acute P14 cells was further downregulated. Likewise, the PD-1 expression on chronic P14 cells also decreased in mice that had cleared the acute LCMV infection, although the level of PD-1 expression on chronic P14 cells at the memory phase of the immune response was significantly higher

than on acute P14 cells ($P = 0.0009$ at day 8 and $P = 0.0018$ at day 30) (Fig. 1c). We also assessed the quantity and level of PD-1 expression on acute and chronic P14 cells in the lymphoid and nonlymphoid tissues of the recipient mice. At day 45 posttransfer, we found that the ratio of acute and chronic P14 cells among total P14 cells in the spleen and liver was similar to the ratio that we observed in the blood (Fig. 1d). Importantly, chronic P14 cells continued to retain a higher constitutive level of PD-1 expression than did acute P14 cells in both lymphoid and nonlymphoid tissues at day 45 posttransfer (Fig. 1e). Hence, while the chronic P14 cells acquired some memory-associated characteristics, such as
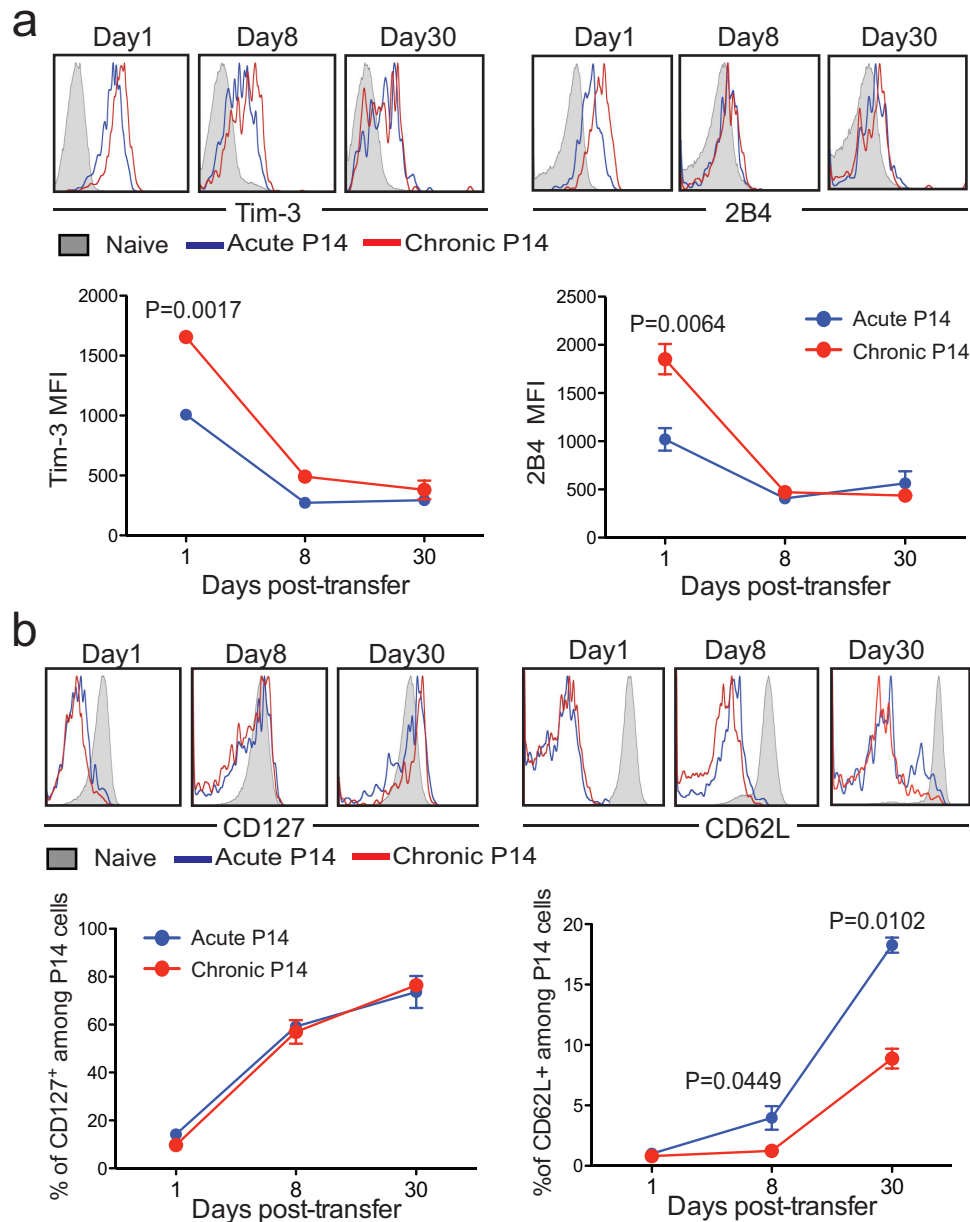
FIG 2 Acute and chronic P14 cells share similar phenotypic properties after transfer into acute immune mice. P14 cells from either LCMV Arm- or CL13-infected mice were transferred into infection-matched (LCMV Arm) recipients at day 8 postinfection. Levels of expression of Tim-3 and 2B4 (a) and CD127 and CD62L (b) on acute and chronic P14 cells were examined at day 1, 8, and 30 posttransfer. Data are representative of those from two or three independent experiments with three to five mice per group. Error bars show SEM.

antigen-independent long-term survival, they also retained an expression program for higher constitutive expression of the PD-1 inhibitory receptor.

After observing the changes in PD-1 expression, we proceeded to more broadly examine the phenotypic changes of chronic P14 cells following rest in the acute immune mice. Interestingly, the downregulated expression of other inhibitory receptors on chronic P14 cells, including Tim3 and 2B4, within the first week upon transfer into the immune environment occurred much faster than the downregulation of PD-1. Furthermore, the level of these inhibitory receptors on chronic P14 cells decreased to similar levels of expression on the acute P14 cells at day 30 posttransfer

(Fig. 2a). We next examined the re-expression of several memory-associated cell surface molecules. After viral clearance, antigen-specific CD8 T cells are known to re-express CD127 and L-selectin (CD62L), which are critical for antigen-independent survival and lymphoid homing, respectively. Surviving chronic and acute P14 cells both upregulated CD127, but the chronic P14 cells were impaired in their ability to re-express L-selectin (CD62L) compared to the acute P14 cells (Fig. 2b).

To determine if the sustained expression of PD-1 in the chronic P14 cells after transfer into acute LCMV mice was associated with decreased functionality, we next assessed the ability of the P14 cells to recall effector cytokine expression. At day 1 post-
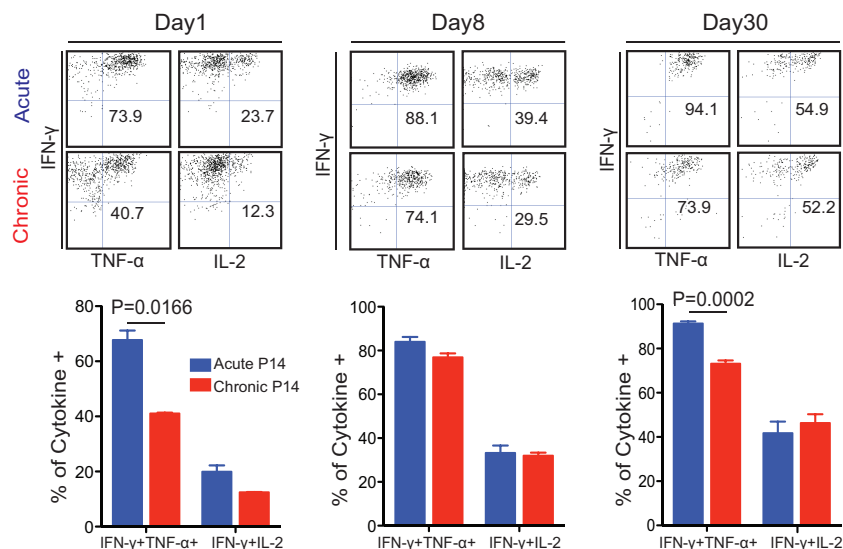
FIG 3 Function of chronic P14 cells was progressively recovered after transfer into acute immune mice. P14 cells from either LCMV Arm- or CL13-infected mice were transferred into infection-matched (LCMV Arm) recipients at day 8 postinfection. Splenocytes of these P14 chimeric mice were stimulated with gp33 peptide for 6 h at days 1, 8, and 30 posttransfer. The percentages of IFN-γ^+ P14 cells able to produce TNF-α or IL-2 were examined. Data are representative of those from three independent experiments with five mice per group. Error bars show SEM.

transfer, the pool of chronic P14 cells was impaired in the ability to express TNF-α and IL-2 compared to acute P14 cells. The impairment in TNF-α and IL-2 expression among the pool of chronic versus acute P14 cells progressively diminished over time, although the chronic P14 cells continued to retain a slight defect in TNF-α expression even after 30 days posttransfer (Fig. 3). While it is well established that exhausted virus-specific CD8 T cells retain elevated levels of PD-1 expression and have an impaired effector function, our data demonstrate that some aspects of the early exhaustion program instilled at the onset of a chronic infection can be stably maintained even after transfer into acute immune mice.

PD-1 promoter demethylation is imprinted in antigen-specific CD8 T cells at the effector phase of chronic infection. We have previously reported that the fully methylated PD-1 promoter of naive CD8 T cells is demethylated during effector differentiation, but it only becomes remethylated in functional memory CD8 T cells that emerge after viral clearance (25). Therefore, we sought to determine if the higher constitutive PD-1 expression of the chronic P14 cells in acute immune mice was coupled to a defect in PD-1 promoter remethylation. To test if PD-1 promoter demethylation was stabilized at the effector phase of T cell exhaustion, we cotransferred day 8 acute and chronic P14 cells into acute infection-matched mice, allowed the cells to rest in the immune environment for 30 days, and then assessed the PD-1 promoter methylation (Fig. 4a). Bisulfite sequencing analysis of the genomic DNA from sorted P14 cells revealed significant remethylation of CpG sites in the PD-1 promoter of acute P14 cells at day 30 posttransfer compared to that in day 8 donor P14 cells (~50% of methylation at day 30 versus ~30% of methylation in day 8 donor cells). However, the PD-1 promoter in chronic P14 cells at day 30 posttransfer remained unmethylated, similar to the day 8 donor P14 cells (Fig. 4b). These data suggest that chronic P14 cells acquire a stable defect in PD-1 promoter DNA remethylation during the effector phase of chronic infection.

Maintenance of the demethylated PD-1 promoter on LCMV-specific effector CD8 T cells from chronically infected mice is antigen independent. To further probe the possibility that minimal TCR signaling from residual viral antigen in recipient mice is responsible for retaining the unmethylated PD-1 promoter on chronic P14 cells, we used the LCMV V35A variant virus, which has a mutated gp33 epitope and is incapable of activating P14 cells (28). Using this model, we cotransferred acute and chronic P14 cells into recipient mice that were acutely infected with LCMV WT or LCMV V35A mutant virus at day 8 postinfection (Fig. 5a). We found that similar to the experiments whose results are shown in Fig. 1c, PD-1 expression on chronic P14 cells underwent a significant reduction after ~1 week in both LCMV WT- and V35A mutant-infected recipient mice, but it then was maintained at a higher constitutive level of expression than on acute P14 cells after 30 days of rest (Fig. 5b and c). Importantly, methylation profiling of P14 cells revealed that the chronic P14 cells still retained a demethylated PD-1 promoter in both WT- and V35A mutant virus-infected recipient mice at day 30 posttransfer, whereas acute P14 cells had remethylated the PD-1 regulatory region (Fig. 5d and e). These data demonstrate that the mechanism for maintenance of PD-1 promoter demethylation in antigen-specific CD8 T cells that is initially generated in response to a chronic infection does not rely on continuous antigen exposure.
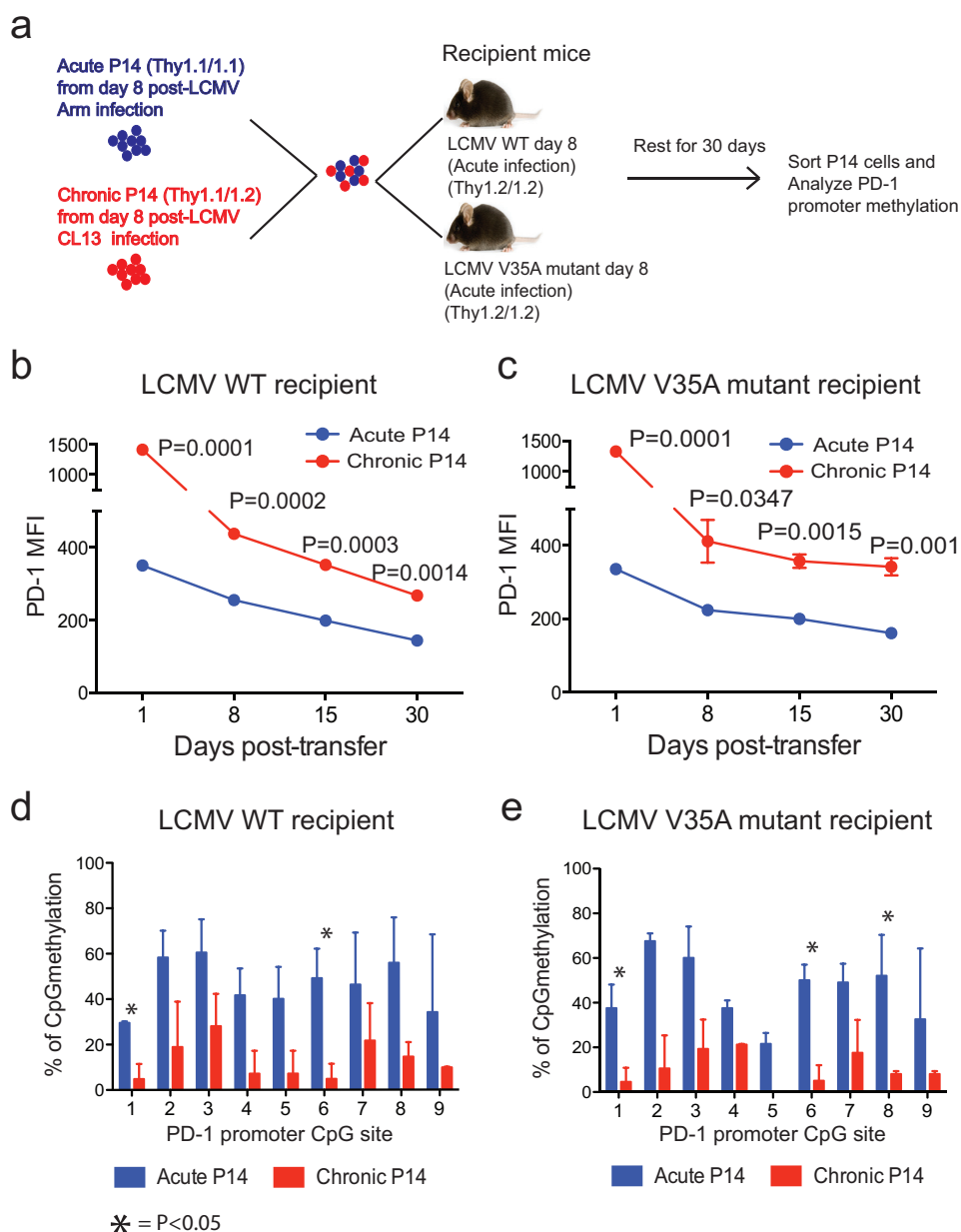
Sustained DNA demethylation of the PD-1 promoter is coupled to impaired T cell expansion during a recall response. Preservation of the demethylated PD-1 promoter along with higher constitutive PD-1 expression suggests that the chronic P14 cells are poised for rapid upregulation of PD-1. To test this, we first stimulated acute and chronic P14 cells with cognate peptide (gp33) *ex vivo* on day 30 posttransfer (Fig. 6a). Indeed, chronic P14 cells expressed a much higher level of PD-1 than did the acute P14 cells upon restimulation, although the levels of induction of PD-1 by gp33 stimulation were comparable between acute and chronic P14 cells (Fig. 6b).
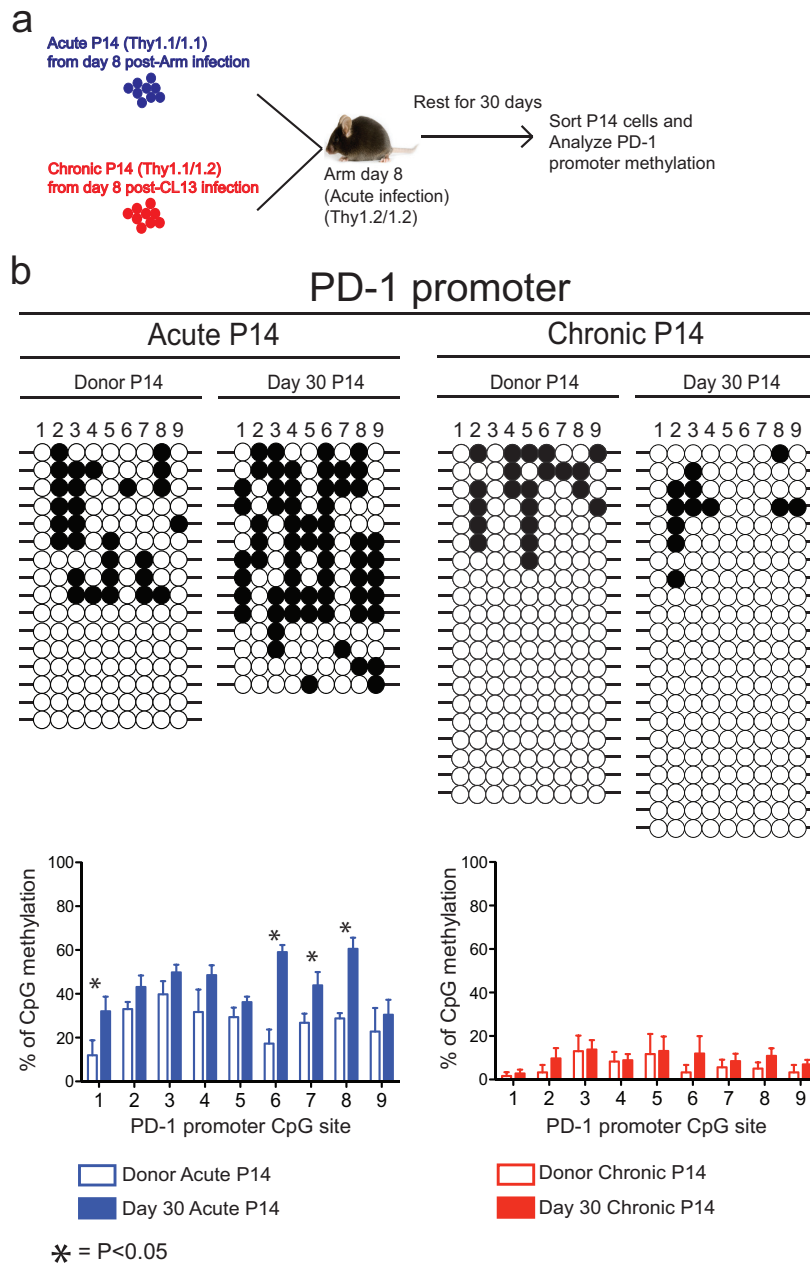
FIG 4 PD-1 promoter DNA demethylation is preserved in chronic P14 cells after transfer into mice that had cleared an acute LCMV infection. (a) Equal numbers of P14 cells from LCMV Arm- or CL13-infected mice were transferred into LCMV Arm infection-matched mice at day 8 postinfection. After 30 days of transfer, acute and chronic P14 cells were subjected to fluorescence-activated cell sorting to analyze DNA methylation. (b) Bisulfite sequencing of PD-1 promoter was performed to analyze DNA methylation on genomic DNA from sorted donor P14 cells (Arm day 8 and CL13 day 8) and sorted acute and chronic P14 cells (day 30 posttransfer). Each line represents an individual clone. The filled circles indicate methylated cytosine, and the open circles indicate unmethylated cytosine. Summary graphs of methylations are shown at the bottom. Data are representative of those from five independent experiments with three to five mice per group. Error bars show SEM.

We next examined the relationship between the poised PD-1 expression and the magnitude of the secondary response. To assess the ability of the chronic P14 cells to mount an *in vivo* recall response, acute and chronic P14 memory cells were sorted at day 30 posttransfer and cotransferred into naive mice. The chimeric mice were then infected with LCMV Arm, and the quantity and quality of the secondary effector and memory cells were measured

(Fig. 7a). At day 8 post-secondary infection, we found that the number of chronic P14 cells was significantly lower than the number of acute P14 cells in the blood (Fig. 7b). While the number of cells available for adoptive transfer precluded us from assessing proliferation kinetics, it is likely that the defect in expansion of chronic P14 cells resulted from impairment in both survival and proliferation capacity of the cells. Longitudinal analysis of the P14

FIG 5 Sustained demethylation of PD-1 promoter on LCMV-specific day 8 P14 cells from chronically infected mice is antigen independent. (a) Equal numbers of P14 cells from Arm- and CL13-infected mice were cotransferred into infection-matched (LCMV WT or V35A mutant day 8) mice. Levels of PD-1 expression of P14 cells were analyzed at days 1, 8, 15, and 30 posttransfer. (b and c) Expression kinetics of PD-1 on acute and chronic P14 cells in blood of WT-infected (b) or V35A mutant-infected (c) mice are shown. (d and e) Methylation of the PD-1 promoter on acute and chronic P14 cells was examined in WT-infected (d) or V35A mutant-infected (e) mice at day 30 posttransfer. Data are representative of those from three independent experiments with three mice per group. Error bars show SEM.

cells revealed that the chronic P14 cells persisted at a lower quantity as they developed into secondary memory cells. We further observed that the chronic P14 cells had a higher level of PD-1 expression than did acute P14 cells during the secondary effector response. Specifically, ~50% of acute P14 cells expressed PD-1, while most of the chronic P14 cells (80 to 90%) expressed PD-1, at day 8 post-secondary infection. Additionally, the level of PD-1 expression was sustained on chronic P14 cells until day 40 post-secondary infection (20 to 30%) (Fig. 7c). Interestingly, both

acute and chronic P14 cells had much slower kinetics for down-regulation of PD-1 expression during the contraction after the recall response than after the primary response. We further observed that the absolute numbers of chronic P14 cells were also much lower than those of acute P14 cells in the spleen and liver at day 40 post-secondary infection (Fig. 7d). Also, the levels of PD-1 expression on chronic P14 cells in blood, spleen, and liver were all significantly higher than on acute P14 cells in these compartments (Fig. 7e). Given that the ratio of chronic P14 cells among total P14
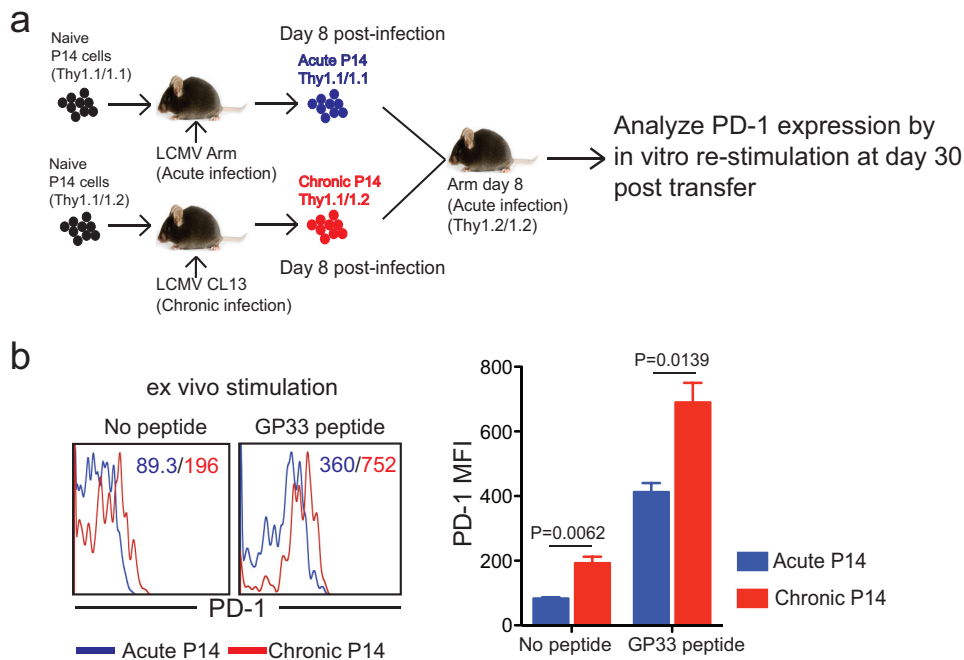
FIG 6 Chronic P14 cells (day 8 effector) rested in acute immune mice for 30 days retain a heightened ability to re-express PD-1. (a) Equal numbers of P14 cells from either LCMV Arm- or CL13-infected mice were cotransferred into infection-matched (LCMV Arm) recipients at day 8 postinfection. (b) After 6 h of stimulation with gp33 peptide, PD-1 expression was analyzed on acute and chronic P14 cells in spleen at day 30 posttransfer. Data are representative of those from two independent experiments with five mice per group. Error bars show SEM.

cells was closely maintained from day 8 to day 40 postinfection (21.3% at day 8 versus 27.1% at day 40), it is likely that the impaired secondary memory formation of chronic P14 cells was due to a defect in expansion during the recall response.

Upon observing a higher level of PD-1 expression on chronic P14 cells than on acute P14 cells, we next examined if expression of other inhibitory receptors was also differentially regulated during secondary response. As before, we transferred an equal number of acute and chronic P14 memory cells into naive mice and then acutely infected the mice with LCMV Arm (Fig. 8a). Again, PD-1 expression on chronic P14 cells was significantly elevated compared to that on acute P14 cells, but surprisingly, the levels of Tim-3 and 2B4 expression on the both chronic and acute P14 cells were comparable at all time points between 8 and 40 days postsecondary infection. We further examined whether higher PD-1 expression was coupled to changes in functionality of chronic P14 cells. We found that at day 40 postinfection, levels of cytokine production among chronic and acute P14 cells were comparable upon restimulation. Interestingly, though, chronic P14 cells had a lower level of granzyme B expression (Fig. 8c). We next proceeded to determine if the higher PD-1 re-expression on chronic P14 cells was responsible for the expansion defect during the recall response. To test this, anti-PD-L1 antibody or control Ig was administered at days 0, 3, 6, and 9 post-secondary infection (Fig. 9a). Indeed, inhibition of the PD-1 pathway led to a significant increase in the number of chronic P14 cells at day 8, and this recovery in the number P14 cells was also maintained until day 35 postinfection, while the number of acute P14 cells was not greatly affected by PD-L1 blockade (Fig. 9b). However, PD-L1 blockade did not result in significant differences in granzyme B expression and cytokine production from either acute or chronic P14 cells at

day 40 after secondary challenge (Fig. 9c). Collectively, these data indicate that stable demethylation of the PD-1 promoter in chronic P14 memory cells facilitates heightened re-expression of PD-1 during a recall response and is broadly coupled to a reduced ability of the cell to undergo expansion during a secondary immune response.

DISCUSSION

Continuous antigen exposure during chronic infection results in sustained expression of the inhibitory receptor PD-1 and a progressive decline in virus-specific CD8 T cell effector function. Our previous study revealed that PD-1 expression on exhausted CD8 T cells was coupled to DNA demethylation of the PD-1 transcriptional regulatory region (25). Interestingly, exhausted CD8 T cells retained the unmethylated PD-1 loci even when the viral load was reduced to undetectable levels at the late phase of mouse and human chronic infection (25, 26). This study generated several important questions: was the retained demethylated state simply due to the persistence of a low level of antigen exposure? If not, when does the demethylation "on" program for PD-1 expression become stably reinforced? In the current study, we found that when antigen-specific CD8 T cells were transferred from day 8 chronic infection into infection-matched immune mice, while the PD-1 surface expression was downregulated, CD8 T cells still maintained an unmethylated PD-1 promoter. One caveat of this experimental setting is that although acute and chronic P14 cells were transferred into the same recipient, their microenvironment inside tissue might be different. For example, chronic P14 cells might preferentially localize to a relatively antigen-rich area compared to acute P14 cells, which could cause the continuous surface expression of PD-1 and stable demethylation of the PD-1 pro-
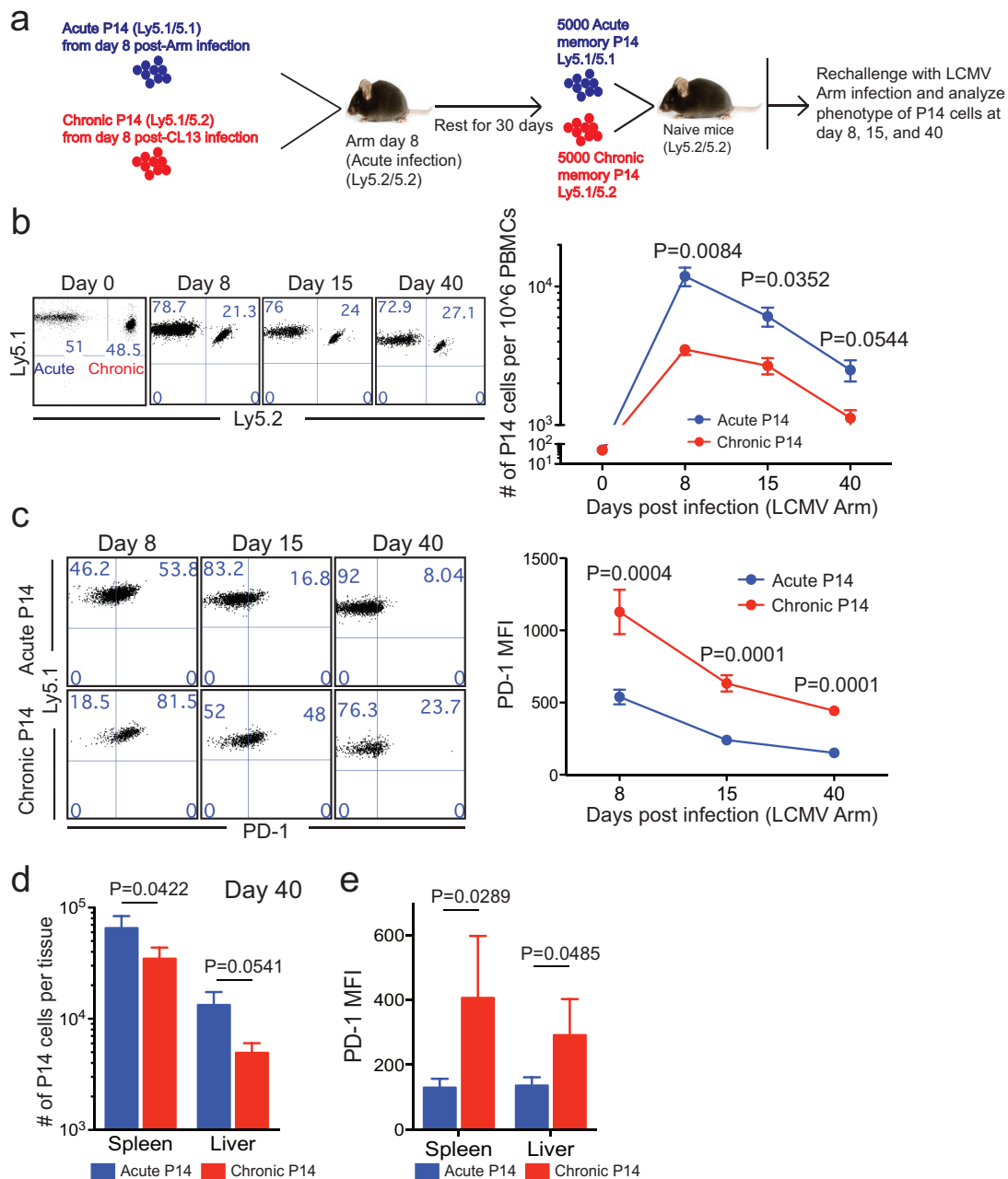
FIG 7 Chronic P14 cells (day 8 effector) rested in acute immune mice for 30 days still show defect in recall response *in vivo*. (a) Equal numbers of acute and chronic P14 cells were sorted at day 30 posttransfer and cotransferred into naive B6 mice, followed by LCMV Arm infection. (b) Flow plots on the left show the ratios of acute and chronic P14 cells in blood at days 0, 8, 15, and 40 post-secondary challenge. Numbers of acute and chronic P14 cells in blood are shown on the right. (c) Levels of PD-1 expression on acute and chronic P14 cells in blood were analyzed at days 8, 15, and 40 post-secondary challenge and are shown on the left. A summary graph of PD-1 expression level is shown on the right. (d) The numbers of acute and chronic P14 cells in spleen and liver were examined at day 40 post-secondary challenge. (e) PD-1 expressions on acute and chronic P14 cells were analyzed at day 40 post rechallenge. Data are representative of those from three independent experiments with five mice per group. Error bars show SEM.

moter. However, we demonstrated that maintenance of PD-1 promoter demethylation does not require a persistent TCR signal, as it still occurred in mice infected with the variant LCMV V35A mutant virus. Thus, these data support a model whereby demethylation of the PD-1 transcriptional regulatory region is imprinted during effector differentiation in response to a chronic infection.

Consistent with the data presented here, it has been previously reported that HIV-specific CD8 T cells from patients who had controlled viral loads by antiviral therapy or naturally, as in the case of HIV elite controllers, also maintained an unmethylated PD-1 promoter even after the viral load was reduced for several years (26). Given that similar results have been obtained with various human and mouse model systems of chronic viral in-
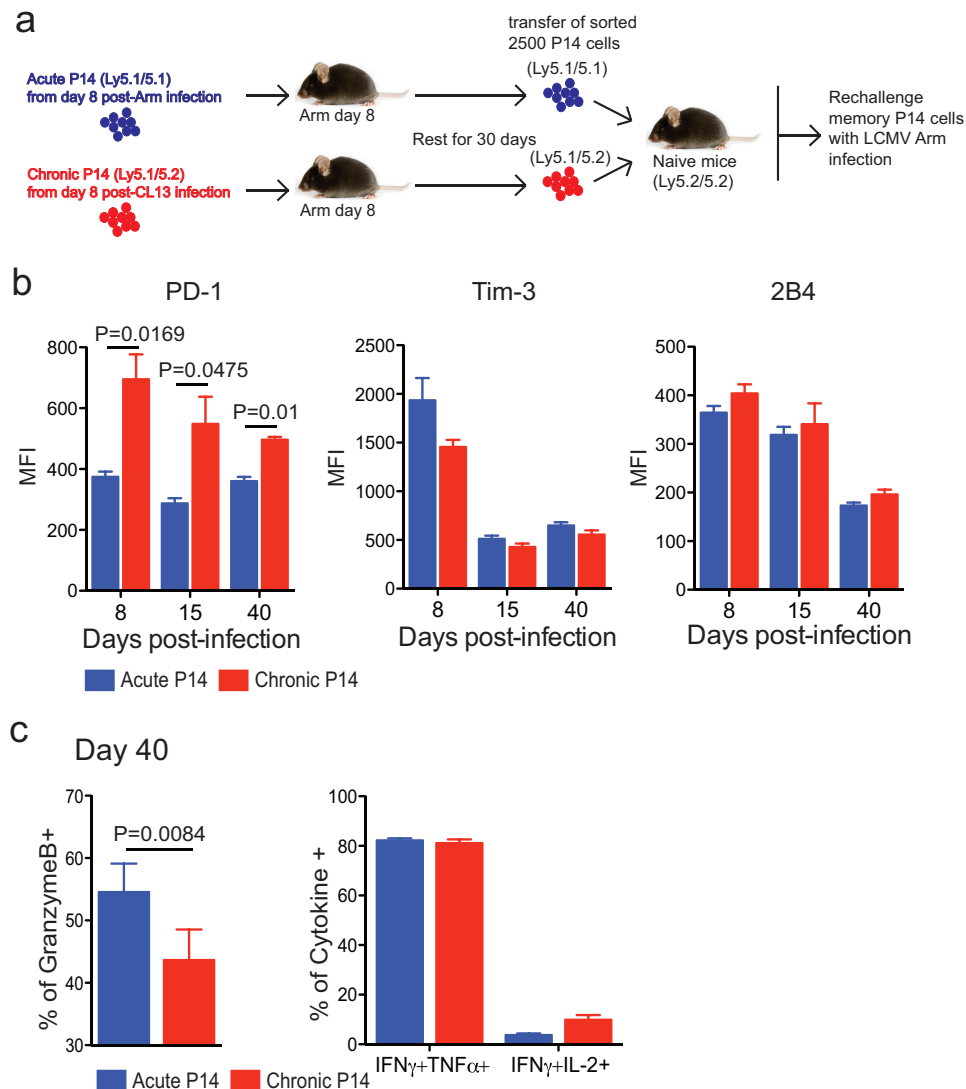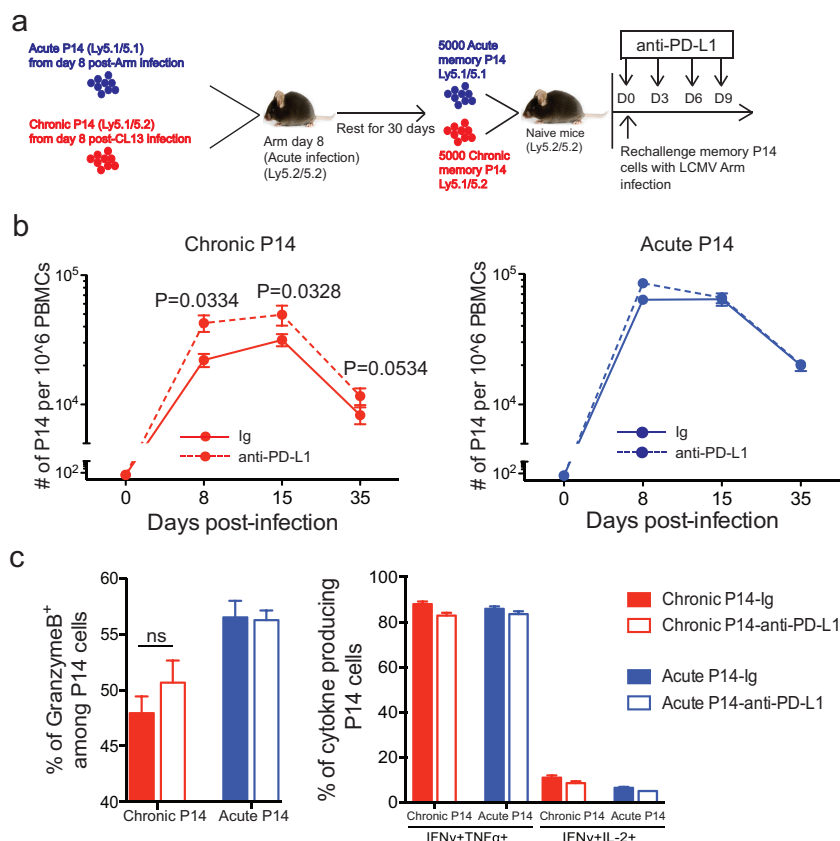
FIG 8 Phenotype and function of acute and chronic P14 cells during recall response. (a) Acute and chronic P14 cells were sorted at day 30 posttransfer and cotransferred into naive B6 mice, followed by LCMV Arm infection. (b) surface expressions of PD-1, Tim-3, and 2B4 were examined on P14 cells in blood at days 8, 15, and 40 post-secondary infection. (c) On day 40 postinfection, granzyme B expression was analyzed on acute and chronic P14 cells in spleen (left). After peptide stimulation for 6 h, cytokine production was examined on acute and chronic P14 cells in spleen (right). Data are representative of those from two independent experiments with five mice per group. Error bars show SEM.

fection, it is tempting to speculate that this may be a general mechanism observed during development of T cell exhaustion and as such may also be observed during the development of cancer. Indeed, continuous exposure to cancer antigen and the inflammatory environments results in CD8 T cell exhaustion, but it remains to be determined if tumor-specific CD8 T cells maintain a demethylated PD-1 promoter after therapy-induced clearance of the cancer. Moreover, future efforts are needed to broadly assess if PD-1 pathway blockade results in long-lived changes in gene expression programs in tumor-specific CD8 T cell following therapy.

Both acute and chronic LCMV infections initially result in demethylation of the PD-1 promoter in virus-specific effector CD8 T cells, yet remethylation of the PD-1 promoter occurs only in CD8 T cells with acute exposure to the virus antigen and in-

flammatory environment. Indeed, T-cell priming prior to day 8 of a chronic infection likely contributes to the differential regulation of PD-1 promoter methylation. Although challenging, future assessment of early priming events will require adoptive transfer of day 5 effector CD8 T cells from acute and chronic infections into infection-matched mice. Such studies will provide additional insight into the roles viral persistence and tropism (29) play in establishing long-lasting epigenetic programs in memory CD8 T cells. In addition to the potential role of viral tropism (differences in antigen-presenting cells), poor CD4 help and higher level of inflammatory cytokines such as type I interferon (30–33) likely also contribute to the initial imprinting of the DNA methylation state during the early stage of chronic infection. Therefore, the combination of longer exposure to antigen and these various signals may culminate in the differential expression or changes in

FIG 9 PD-L1 blockade improves the clonal expansion of chronic P14 cells transferred into acute immune mice during secondary challenge. (a) P14 cells from either LCMV Arm- or CL13-infected mice were transferred into infection-matched (LCMV Arm) recipients at day 8 postinfection. Then an equal number of acute and chronic P14 cells were sorted on day 30 posttransfer and then transferred into naive B6 mice, followed by LCMV Arm infection. Anti-PD-L1 antibody or control Ig was administered to mice at days 0, 3, 6, and 9 after rechallenge. (b) Numbers of acute (right) and chronic (left) P14 cells in blood of control Ig- or anti-PD-L1-treated mice during secondary challenge. (c) Thirty-five days after infection, granzyme B expression was analyzed on acute and chronic P14 cells in spleens of control Ig- and anti-PD-L1-treated mice (left). After peptide stimulation for 6 h, cytokine production on acute and chronic P14 cells was examined in spleens of control Ig- and anti-PD-L1-treated mice (right). Data are representative of those from two independent experiments with five mice per group. Error bars show SEM.

specificity of the DNA methyltransferases involved in epigenetic modification.

In a previous study using the same model system as ours, it was reported that antigen-specific effector CD8 T cells isolated from chronically LCMV-infected mice on day 5 or 8 could escape from exhaustion if they were removed from continuous antigen exposure (34, 35). Angelosanto et al. showed that these effector CD8 T cells arising from chronic infection were maintained over time, downregulated PD-1, and re-expressed CD127 in infection controlled recipients, which was consistent with our data (35). The authors concluded that partially exhausted virus-specific CD8 T cells retained the potential to differentiate into memory CD8 T cells. While we have several overlapping observations, our phenotypic and epigenetic analyses revealed that the virus-specific effector CD8 T cells derived from chronic infection are restricted in their ability to develop memory T cell properties. First, chronic P14 cells undergo a severe contraction within a week of adoptive transfer into acute immune mice, which results in a reduced quantity of memory T cells. Second, re-expression of the homing molecule L-selectin (CD62L) on chronic P14 cells was limited compared to that on acute P14 cells during memory differentiation. Third, and most important, the demethylated transcriptional reg-

ulatory region of PD-1 on memory CD8 T cells arising from chronic infection-derived effector T cells was coupled to accelerated PD-1 expression upon restimulation. Prior analyses of exhausted T cells transferred and rested in naive mice for 56 days also revealed higher PD-1 expression than in memory CD8 T cells upon secondary acute infection until day 30 (36). Our results presented here provide a possible mechanism of the stable poisoning of PD-1 expression in exhausted CD8 T cells. This poised PD-1-mediated inhibitory signal appeared to impair the production of cytokines following *ex vivo* restimulation and ultimately reduced the clonal expansion of chronic P14 cells during a recall response. Lastly, the expansion defect was restored by PD-1 signal blockade, confirming that PD-1 was responsible for this defective recall response. Prior studies investigating PD-1 blockade during a recall response of memory CD8 T cells generated from vaccinia virus with and without CD4 help and expressing higher PD-1 than CD4-helped memory CD8 cells also noted that the expansion defect of helpless memory CD8 T cells could be restored by PD-1 blockade during the recall response (37). Although the mechanism was linked to a higher level of PD-1 expression in the helpless memory cells, it is unclear if it is also due to the epigenetic regulation of PD-1 promoter. Therefore, although effector CD8 T cells

derived from chronic infection retain the ability to re-express several surface markers associated with memory T cell differentiation, effector T cells from a chronic environment may be epigenetically limited in regaining the capacity to differentiate into fully functional memory CD8 T cells.

In summary, we determined that the priming phase of chronic infection is critical for instilling a stable PD-1 DNA methylation program in antigen-specific CD8 T cells, and these data provide a mechanism to explain why memory CD8 T cells derived from a chronic infection may retain a functional impairment. These results shed new insight into the molecular mechanisms that govern the generation of long-lived functional and nonfunctional memory T cells.

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